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13. ABSTRACT *(Maximum 200 Words)*

This study addresses the initiating causes and early events of breast cancer. Increased oxidative DNA damage has been reported in breast tissue from breast cancer patients. This study investigated if the cytochrome P450 enzyme CYP1B1 plays a role in the formation of oxidative damage. The CYP1B1 enzyme is involved in the metabolic activation of environmental carcinogens, and also metabolizes estrogen to a carcinogenic metabolite. The concept tested was that high levels of CYP1B1 lead to increased oxidative damage, thereby initiating the carcinogenic process. CYP1B1 expression and oxidative damage were determined in 58 breast tissue specimens from reduction mammoplasties and non-tumor peripheral tissue from mastectomies. CYP1B1 expression was determined by real time RT-PCR relative to the β-actin gene. 8-hydroxy-2'deoxyguanosine (8-oxoG), a reliable index of overall oxidative DNA damage, was determined by 32P-postlabeling. 8-oxoG levels varied thirty-fold and CYP1B1 expression 500-fold among specimens. In the limited number of specimens analyzed, 8-oxoG levels were slightly higher in specimens from controls then specimens from cases. CYP1B1 expression differed between cases and controls only in women over 50 years. No correlation between CYP1B1 expression and 8-oxoG levels was observed.

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FOREWORD

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Regine Goth-Goldstein 7/30/02

PI Signature Date

Table of Contents

Oxidative damage, CYP1B1 and Breast Cancer

Introduction

Oxidative damage to DNA has been shown to occur *in vivo* indicating that DNA can be damaged as a consequence of normal metabolism (1). Because a large number of DNA base modifications are caused by oxidative stress, it is difficult to measure the whole spectrum of modified lesions. One of the more abundant lesions, 8-hydroxy-2'deoxyguanosine (8-oxoG), which is known to cause a G to T transversion, is used as a reliable index of overall oxidative DNA damage. DNA bases modified by oxidative stress can lead to mutations, chromosomal abnormalities and altered gene expression.

Continuous oxidative stress, which leads to the formation of free radicals from molecular oxygen, has been implicated in various stages of chemical carcinogenesis (1). The etiology of the majority of human breast cancers is not understood. Evidence supporting a link between oxidative damage and breast cancer includes the finding that oxidative DNA damage is increased in breast tissue from cancer patients compared to controls (2) and that cells with BRCA1 deficiencies lack transcription-coupled repair of oxidative damage (3). Suggested breast cancer risk factors such as exposure to environmental carcinogens, dietary fat intake and estrogen metabolism could all act through an oxidative stress mechanism. The oxidative metabolism of environmental contaminants, like polycyclic aromatic hydrocarbons (PAHs) by the cytochrome P450 enzymes CYP1A1 and CYP1B1, creates reactive oxygen species that result in oxidative damage to DNA. The amount of oxidative damage far exceeds that of direct PAH adduct formation (4). Endogenous sources of oxidative damage include the oxidative metabolism of steroid hormones, such as estrogen. Estradiol is converted to catechol estrogens through hydroxylation by various cytochrome P450 isozymes, leading predominantly to formation of 2-hydroxyestrogen (5). Only the isozyme CYP1B1 catalyzes the predominant formation of 4-hydroxyestrogen (4-OH-E2). It has been suggested that estrogen exerts some of its effects in target tissues like breast through locally formed estrogen metabolites. The estrogen metabolite 4-OH-E2 might fulfill such a role (6). 4-OH-E2 has also been implicated in estrogen-associated cancers in various rodent models and is known to elicit an oxidative stress response induced by free radicals generated by metabolic redox cycling reactions (6).

We have found previously that *CYP1B1* is expressed in breast and that expression levels were significantly higher in non-tumor breast tissue from breast cancer patients than from healthy individuals, suggesting a possible role of CYP1B1 in the etiology of breast cancer. The concept investigated here is that high levels of *CYP1B1* lead to increased oxidative damage, thereby initiating the carcinogenic process.

Body of Final Report

A collection of histologically normal breast tissue specimens from mastectomy patients and from reduction mammoplasties has been analyzed both for expression levels of the *CYP1B1* gene and for markers of oxidative damage.

Specimens: A tissue bank established by Peralta Cancer Center in 1981-1988 and maintained by Aeron Biotechnology Inc. was obtained. Only histologically normal breast tissue specimens (as determined by pathology report) were used for these studies. The specimens had been dissected and isolated from adipose and connective tissue, so that only epithelial material was stored frozen as organoids (8). The control group consisted of 18 reduction mammoplasty specimens (Table 1). The case group consisted of 38 peripheral non-tumor tissue specimens obtained from mastectomy patients and 1 tissue specimen contralateral to the tumor. The average age of the control group is 18 years younger than the case group and is a potential confounder in the statistical analysis of the specimen. Stratifying the groups by age solves this problem, but reduces the sample size considerably.

Table 1: Description of specimen.

Groups	*Specimen Analysed (n)*	*Average Age (y)*
Controls	18	43.1
Controls ≥50y	5	64.0
Cases	38	61.1
Cases ≥50y	31	65.8
Total	56	55.3

Measuring CYP1B1 expression levels: Total RNA was isolated from 56 breast epithelial specimens using TRI Reagent as recommended by the manufacturer. Two μg of total RNA was reverse transcribed at 37°C for 1 hour using $(\text{oligo dT})_{16}$ and Moloney murine-leukemia virus reverse transcriptase. The cDNA was stored as several small aliquots at -80°C.

A PCR-based assay for calibrator-normalized relative quantitation was developed using a rapid micro-capillary cycler with real-time product detection by fluorescence (LightCycler, Roche Molecular Biochemicals). The cDNA was amplified with β-actin primers (9) and with CYP1B1 primers (10, 11). Both primer sets are designed to span an intron, thus excluding amplification of any contaminating genomic DNA and generated products of 158 bp for CYP1B1, and 273 bp for β-actin. cDNA from primary cultures of a human mammary epithelial cell line (HMEC184) was included in each PCR reaction as a calibrator. CYPIB1 levels are expressed as a ratio to a reference gene: β-actin. This corrects for sample variations in the amount and quality of the isolated RNA. The ratio of CYP1B1/β-actin for the sample is divided by the CYP1B1/β-actin ratio for the calibrator

in order to correct for run-to-run variations and most importantly, differing amplification efficiencies between the two primer sets. The reliability of the assay to measure *CYP1B1* expression was determined by re-analyzing 10% of the cDNA specimens. The CYP1B1 levels had a variance of 12%.

Comparison of CYP1B1 expression in breast cancer patients and controls: In breast tissue specimens from cases and controls, there was a 500-fold variation in the *CYP1B1* expression level (range 0.05 to 25.87). The arithmetic means of the CYP1B1/β-actin ratio of the cases and controls (see Table 2) were compared using a two-tailed student's t-test. No significant difference in *CYP1B1* levels between the two groups was found ($p=0.7770$). In a previous study, we did discover a statistically significant difference ($p = 0.0473$) in CYP1B1 values between case and control groups but the sample size was much larger (32 controls and 24 cases) (14). This finding did not repeat in this smaller set of samples. When the two groups are stratified by age (< 50 and ≥ 50 years), which roughly correspond to menopausal status, the p-value of the group over 50 years is 0.0681, a significant result if the size of the control group over 50 years was larger. Also, the range of CYP1B1 levels was noticeably more restricted in controls over 50, possibly indicating that endogenous estrogen levels might influence *CYP1B1* expression.

Table 2: Statistical summary of *CYP1B1* gene expression levels in non-tumor breast tissue of breast cancer patients and healthy controls.

	CYP1B1/actin ratio		
Groups	*Mean (SD)*	*Median*	*Range*
Controls	2.28 (4.05)	0.84	13.57-0.05
Controls ≥50y	0.98 (0.55)	1.02	1.69-0.23
Cases	2.65 (5.30)	0.77	25.87-0.13
Cases ≥50y	3.02 (5.79)	0.93	25.87-0.13
Total	2.53 (4.90)	0.80	25.87-0.05

A scatter plot of CYP1B1 expression levels of all specimen and age shows no correlation (Figure 1, appendix). The Pearson's product moment correlation coefficient was determined to be $r = 0.0918$.

Determination of oxidative damage:
DNA was isolated from the 56 breast specimens by the newly developed sodium iodide method that has been shown to minimize, if not eliminate, the oxidative damage to DNA that occurs during isolation (12). The 8-oxoG content (a reliable index of overall oxidative DNA damage) was determined by 32P-postlabeling in collaboration with Dr. Donghui Li, UT, MD Anderson Cancer Center (13).

Comparison of oxidative damage in breast cancer patients and controls: The relative 8-oxoG levels varied about 30-fold among the 56 specimens tested (Table 3). A comparison of the mean oxidative damage level between cases and controls (using a two-tailed

student's t-test) shows a significant difference between the two groups (p = 0.0681) with oxidative levels of DNA being higher in the control group than the cases. A scatter plot of oxidation levels and age of all specimen (Figure 2, appendix) does indicate a slight correlation that is inversely related to age (Pearson's product moment correlation coefficient r = -0.2585). When the two groups are stratified by age, the difference in oxidative damage found in the older groups is no longer significant (p-value = 0.4810).

Table 3: Statistical summary of oxidative damage in non-tumor breast tissue of breast cancer patients and healthy controls.

	8-oxoG/dG ($x10^6$)		
Groups	*Mean (SD)*	*Median*	*Range*
Controls	137.5 (43.7)	131.6	206.1-73.4
Controls ≥50y	130.5 (47.0)	151.5	181.1-74.8
Cases	113.5 (46.4)	119.5	190.6-6.60
Cases ≥50y	108.3 (45.4)	112.3	190.6-6.60
Total	121.2 (46.5)	120.0	206.1-6.60

Comparison of oxidative damage and CYP1B1 expression levels between breast cancer patients and controls: The distribution of oxidative damage levels over the range of CYP1B1 levels appears to be normal (Figure 3, appendix). In specimen with high *CYP1B1* expression (ratio > 5), the oxidative damage appears also to be normally distributed and not dependent on high expression of the *CYP1B1* gene. Overall, these results on *CYP1B1* expression and oxidative damage do not support our hypothesis that higher levels of CYP1B1 leads to increased oxidative damage.

Future goals

We plan to determine the genetic polymorphism of the CYP1B1 gene in the specimens to determine if a certain CYP1B1 genotype correlates with oxidative damage levels. We will also determine the PAH DNA-adduct levels to evaluate the relationship of PAH exposure to oxidative damage levels.

Key Research Accomplishments

- A sensitive and reliable RT-PCR assay was developed to determine expression of *CYP1B1*by real-time PCR.
- A 500-fold variation in expression of *CYP1B1* in 56 non-tumor breast tissue samples was determined.
- More breast cancer cases over 50 years old than controls in that age group showed high *CYP1B1* expression.
- Oxidative damage was measured in the same 56 specimens and was found to vary by a factor of 30 with mean 8-oxoG levels slightly higher in controls than cases.
- No correlation between CYP1B1 expression and oxidative damage could be observed.

Reportable Outcomes

Abstracts

An abstract on this study was submitted for presentation at the Era of Hope, Department of Defense Breast Cancer Research Program Meeting in September 2002.

Conclusions

Because of the potential important role of CYP1B1 in the activation of environmental and endogenous compounds to carcinogenic intermediates, it was hypothesized that high *CYP1B1* expression could result in high levels of oxidative damage. We found a very large inter-individual variation in *CYP1B1* expression and a much smaller variation in levels of oxidative damage. No correlation between *CYP1B1* expression and oxidative damage was observed.

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List of personnel receiving pay from the research effort

Regine Goth-Goldstein, Principal Investigator

Marion Russell, Senior Research Associate

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Appendix Cover Sheet

List of content:

Figure 1. CYP1B1 Gene Expression in Non-Tumor Breast Tissue and Age of Donors.

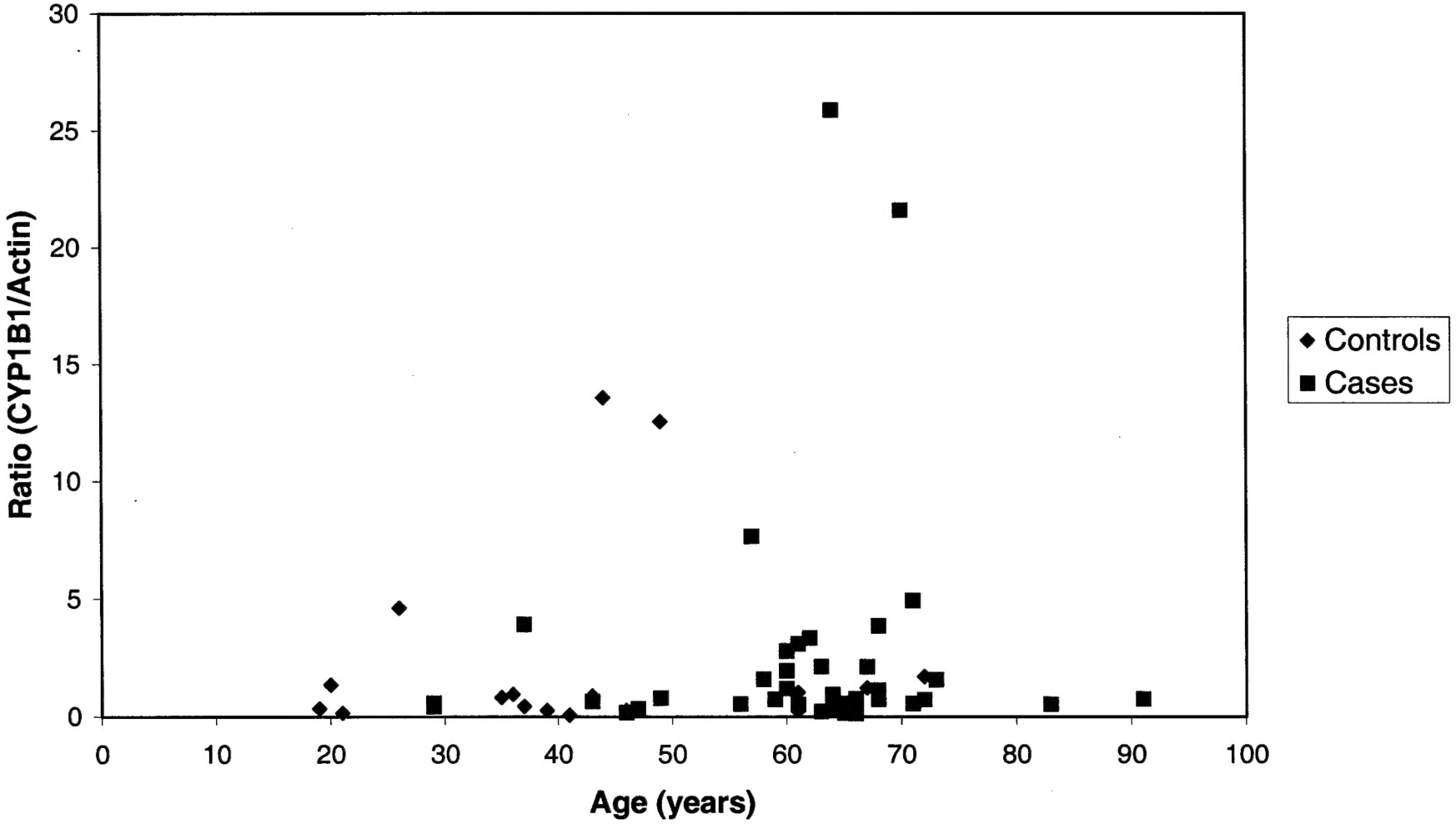

Figure 2. Oxidative Damage Measured in Non-tumor Breast Tissue and Age of Donors.

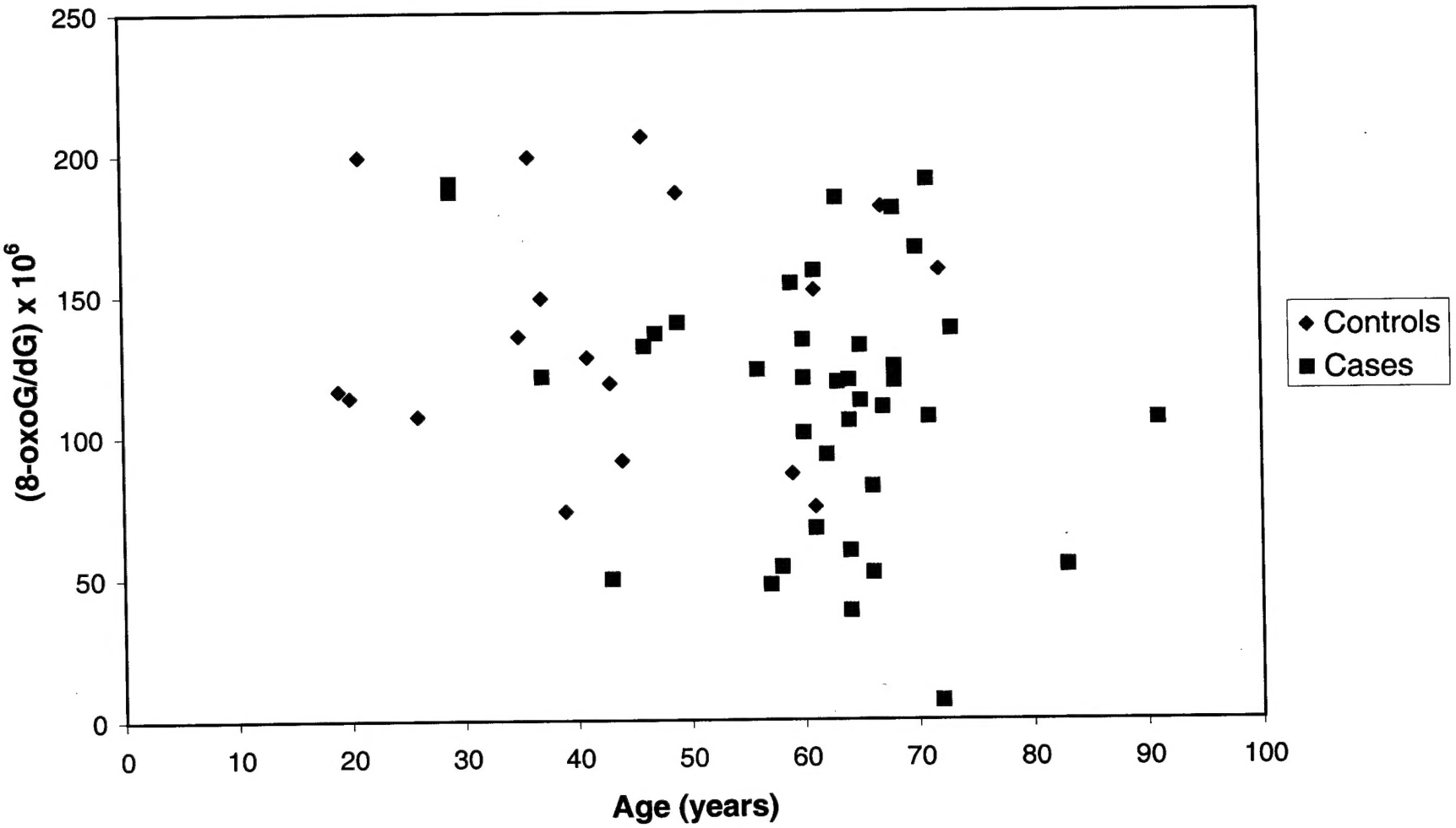

Figure 3. CYP1B1 Expression Levels as a Function of Oxidative Damage in Non-tumor Breast Tissue of Cases and Controls.